Molecular characterization of the erythromycin resistance plasmid pPV142 from *Staphylococcus simulans*

Abstract

The 2.5-kb erythromycin resistance (Em^R) plasmid pPV142 of *Staphylococcus simulans* 13044 was isolated and characterized. Sequence analysis identified ORF1 and ORF2 encoding a 158-residue replication protein (Rep142) and a 244-residue erythromycin resistance protein (Erm, rRNA adenine *N*-6-methyltransferase), respectively. Structural analysis and Southern hybridization showed that the *rep* and *ermM* genes in pPV142 shared homology with the Em^R plasmid pPV141 (2.4 kb) of *S. chromogenes* 3688 and other Em^R plasmids known to exist in staphylococci and bacilli. Based on the presence of a 61-bp repeat upstream of the *ermM* gene, pPV142 is apparently a unique member of the pSN2 family of Em^R plasmids able to express erythromycin resistance constitutively.

Keywords: *Staphylococcus simulans* plasmid; Erythromycin resistance plasmid

1. Introduction

Staphylococcus simulans, *S. chromogenes* (formerly *S. hyicus* ssp. *chromogenes*, [1]), *S. hyicus*, and *S. epidermidis* are commonly found in domesticated animals (cattle and swine) and may be involved as opportunistic pathogens in the pathology of epidermitis, otitis and mastitis [2–4]. Following the isolation of plasmids from *S. hyicus* [4] several antibiotic resistance plasmids were identified [5]. So far, plasmids of *S. hyicus* encoding resistance to chloramphenicol [6], tetracycline [7,8], streptomycin [9,10], and erythromycin [11,12], and the erythromycin resistance plasmid of *S. chromogenes* [13] have been studied in detail. On the other hand, the plasmid biology of *S. epidermidis* and *S. simulans* from animal sources has not been explored.

Molecular characterization of antibiotic resistance plasmids in coagulase negative staphylococci associated with animals provides information on relatedness among resistance plasmids isolated from different sources. Information on these plasmids may also be useful in the development of cloning vectors with application in the genetic engineering of other Gram-positive microbes.

We have previously reported on the molecular properties of pPV141, a 2.4-kb erythromycin resistance (Em^R) plasmid present in *S. chromogenes* 6388

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[13] and the use of *erm* as a reporter gene in vector constructs [14,15]. In this paper, we describe the nucleotide sequence and structural features of pPV142, a 2.5-kb plasmid from *S. simulans* 13044, and compare its molecular properties with several known Em^R plasmids.

2. Materials and methods

2.1. Microbial strains and maintenance

The coagulase negative and erythromycin resistant *S. simulans* 13044 (otitis, cow) was classified with 93%+ accuracy by the API STAPH Track kit (API Laboratory Products, Ltd., St. Laurent, Quebec) and supplied by the Purdue University School of Veterinary Medicine (West Lafayette, IN). Control cultures with Em^R plasmids included *S. epidermidis* (pE131, gift from J.T. Parisi), *S. aureus* (pE194, gift from B. Weisblum), and *S. chromogenes* (pPV141, 13). Cultures were grown at 37°C for 24 h in tryptic soy broth (TSB, Difco Laboratories, Detroit, MI) with erythromycin at 15 μg ml^{-1}. Cultures were stored at 4°C between weekly transfers.

2.2. Plasmid analysis

Procedures used in plasmid isolation and curing were previously described [13]. Plasmids present in the original *S. simulans* 13044 strain and cured cultures were analyzed and the putative Em^R plasmid was identified by agarose gel electrophoresis (AGE) in 0.7% agarose (FMC Corporation, Rockland, ME) in Tris/borate/EDTA buffer system (0.089 M Tris base, 0.089 M boric acid, 0.002 M EDTA, pH 8.3), at 100 V for 4 h.

2.3. DNA analysis and manipulations

The putative Em^R plasmid of *S. simulans* 13044 was removed from agarose gels with GenElute™Minus EtBr Spin Columns (Supelco, Bellefonte, PA) and further purified by CsCl density gradient centrifugation [16] or by Elutip-d treatment (Schleicher and Schuell, Keene, NH). A restriction map was constructed by single and coupled digestions with an array of restriction endonucleases (BRL Life Technologies, Gaithersburg, MD), under conditions recommended by the manufacturer. DNA fragments were analyzed in 1.2% agarose gels under conditions described above.

The position of the *erm* region was approximated by cloning restriction endonuclease fragments into pBR322 with T4 DNA ligase using the *Cla*I and *Hin*dIII insertion sites following recommendations of the vendor (United States Biochemical, Cleveland, OH). Ligation mixtures were used to transform freshly prepared competent cells [17] of *Escherichia coli* DB11, a highly Em-sensitive variant of *E. coli* K-12 (gift from J. Davies). Em^R clones were detected on Luria-Bertani (LB) agar plates (1% tryptone, 0.5% each of yeast extract and NaCl, and 1.5% agar) supplemented with ampicillin (100 μg ml^{-1}) and erythromycin (25 μg ml^{-1}), after incubation for 48 h at 37°C.

Biotinylated probes were prepared by a standard method [18] from the ca. 1.3-kb *Hin*dIII/*Taq* fragment of pPV141 from *S. chromogenes* [13], the 1.4-kb *Taq*I fragment of pE194 from *S. aureus* [19] and the 1.6-kb *Hin*dIII/*Taq*I fragment of pNE131 from *S. epidermidis* [20], which delineate the *erm* region of these plasmids. Southern hybridizations with the putative Em^R plasmid pPV142 of *S. simulans* 13044 digested with *Taq*I were carried out at 45% formamide concentration, in an Automated Southern Blot System (Oncor, Gaithersburg, MD), according to the manufacturer's recommendations.

Restriction endonuclease fragments of pPV142 were cloned into the multiple cloning site on pUC19 and competent *E. coli* DH5α (BRL Technologies, Gaithersburg, MD) were transformed with the ligation products. Recombinant (white) clones were selected on LB agar (see above) supplemented with 100 μg ml^{-1} ampicillin and 50 μg ml^{-1} X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside). Recombinant plasmids were isolated and purified in a CsCl gradient. DNA sequencing was based on the dideoxynucleotide chain termination method [21] and carried out in triplicate in an ALF DNA Sequencer unit (Pharmacia, New Brunswick, NJ), with a T7 Autoread Sequencing Kit using M13 universal and M13 reverse primers. Putative −10, −35 and Shine-Dalgarno sequences were located with the aid of the Clone Manager Program Version 4 (Scientific and Educational Software, State Line, PA).

Comparison of the sequences of pPV142 and other Em^R plasmids was based on analysis with BLASTP and BLASTX database programs [22]. Multiple sequence alignments was done with the aid of DNASIS™ WINDOWS 2.1 (Hitachi Software Engineering America, San Bruno, CA).

2.4. Nucleotide accession number

The DNA and deduced amino acid sequences have been deposited in GenBank under the accession number AF019140.

3. Results and discussion

3.1. Plasmid composition

Three plasmids with molecular masses corresponding to ca. 2.5 kb, 4.3 kb and 40 kb were detected by AGE analysis in *S. simulans* 13044. The smaller plasmids were similar in size to plasmids found in other

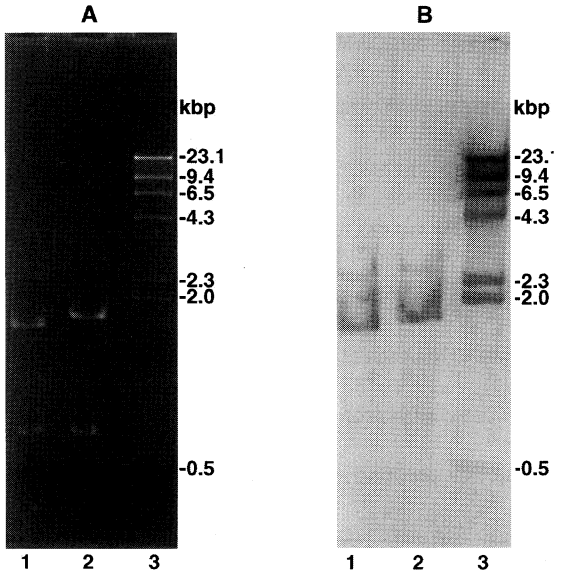

Fig. 1. Agarose gel electrophoretic (A) and Southern hybridization (B) patterns of *Taq*I-digested pPV142 with a biotinylated *Hin*dIII/*Taq*I fragment of pPV141 as the probe; lane 1: pPV141; lane 2: pPV142; lane 3: *Hin*dIII-digested lambda-DNA control (probed with biotinylated lambda fragments). Identical results were obtained with biotinylated *Taq*I fragments from pE194 and pNE131 as probes.

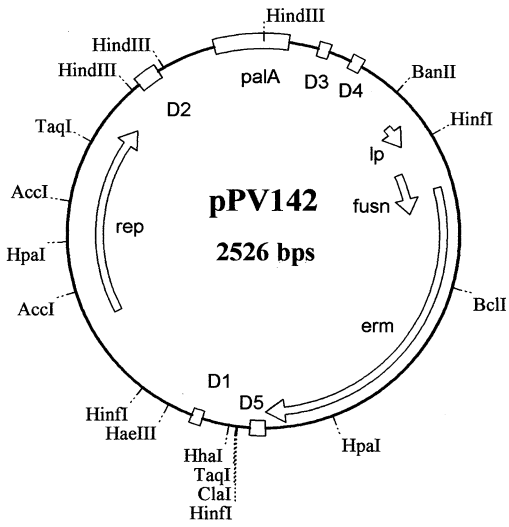

Fig. 2. Restriction endonuclease map of pPV142 from *S. simulans* 13044. Boxes indicate the minus-origin (M-O) palindromic sequence (*palA*), and areas of dyad (D) symmetry. Arrows designate the ORF1 (*rep*), ORF2 (*erm*), leader peptide (lp) and *erm*-fusion (fusn) regions.

coagulase negative staphylococci [4,5], although the presence of the 40-kb plasmid was atypical for this species.

Exposure of *S. simulans* 13044 to ethidium bromide resulted in the loss of the Em^R phenotype at a frequency of 3.0×10^{-2}, and the disappearance of the 2.5-kb (pPV142) band from the plasmid profile.

The identity of pPV142 as an Em^R plasmid was confirmed by subcloning various restriction endonuclease fragments into pBR322 and using the constructs to transform competent *E. coli* DB11. Transformed DB11 clones with erythromycin resistance up to 100 μg ml^{-1} concentration were further analyzed. The Em^R phenotype was associated with the cloning of a ca. 1.3-kb *Hin*dIII/*Cla*I (coordinates 0–1.3) fragment of pPV142. Strong signals in Southern probes of *Taq*I-digested pPV142 with biotinylated fragments (*erm*) of pNE131 (*S. epidermidis*), pE194 (*S. aureus*), and pPV141 (*S. chromogenes*) indicated homology with these staphylococcal Em^R plasmids (Fig. 1).

3.2. Molecular properties of pPV142

The restriction endonuclease map and complete nucleotide sequence of pPV142 are shown in Figs.

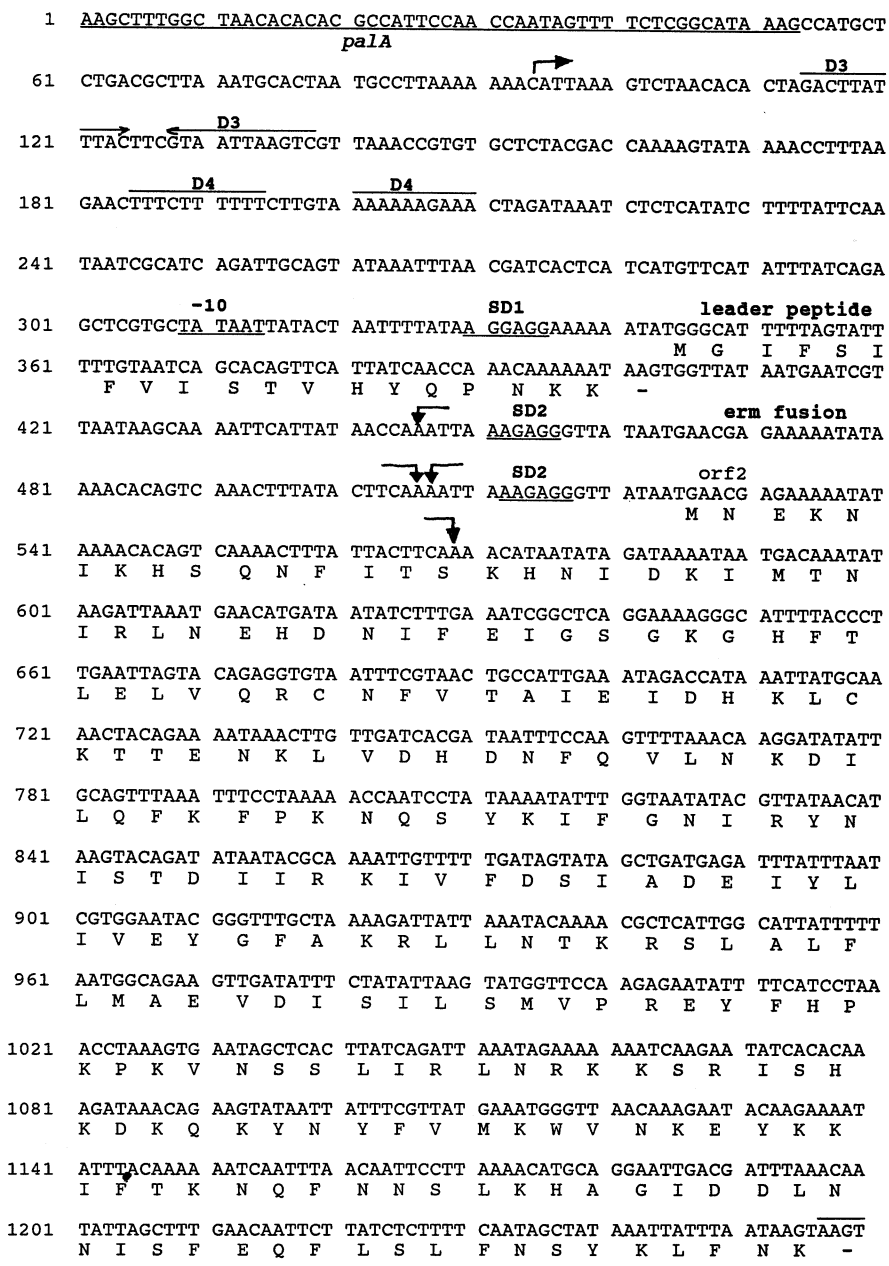

Fig. 3. Complete nucleotide sequence of pPV142 numbered from the *Hin*dIII site shown as the zero coordinate 1. Homologies with pE194 (►) and pSN2 (►►), the 61-bp repeat sequence (▼), and open reading frames (ORF1 and ORF2) with the amino acid sequences of putative polypeptides are labeled. The putative promoter hexamer −10 and ribosome binding (Shine-Dalgarno, SD) sites are underlined. Converging arrows indicate dyad symmetries (D).

2 and 3. The plasmid was 2526 bp long and had two major ORFs separated by a 441-bp spacing. The larger ORF2, extending from coordinates 524 to 1258 (reading frame 2), was the putative *erm* gene and encoded a 244-amino acid protein. The gene product of *erm* in pPV142 shared a high degree of

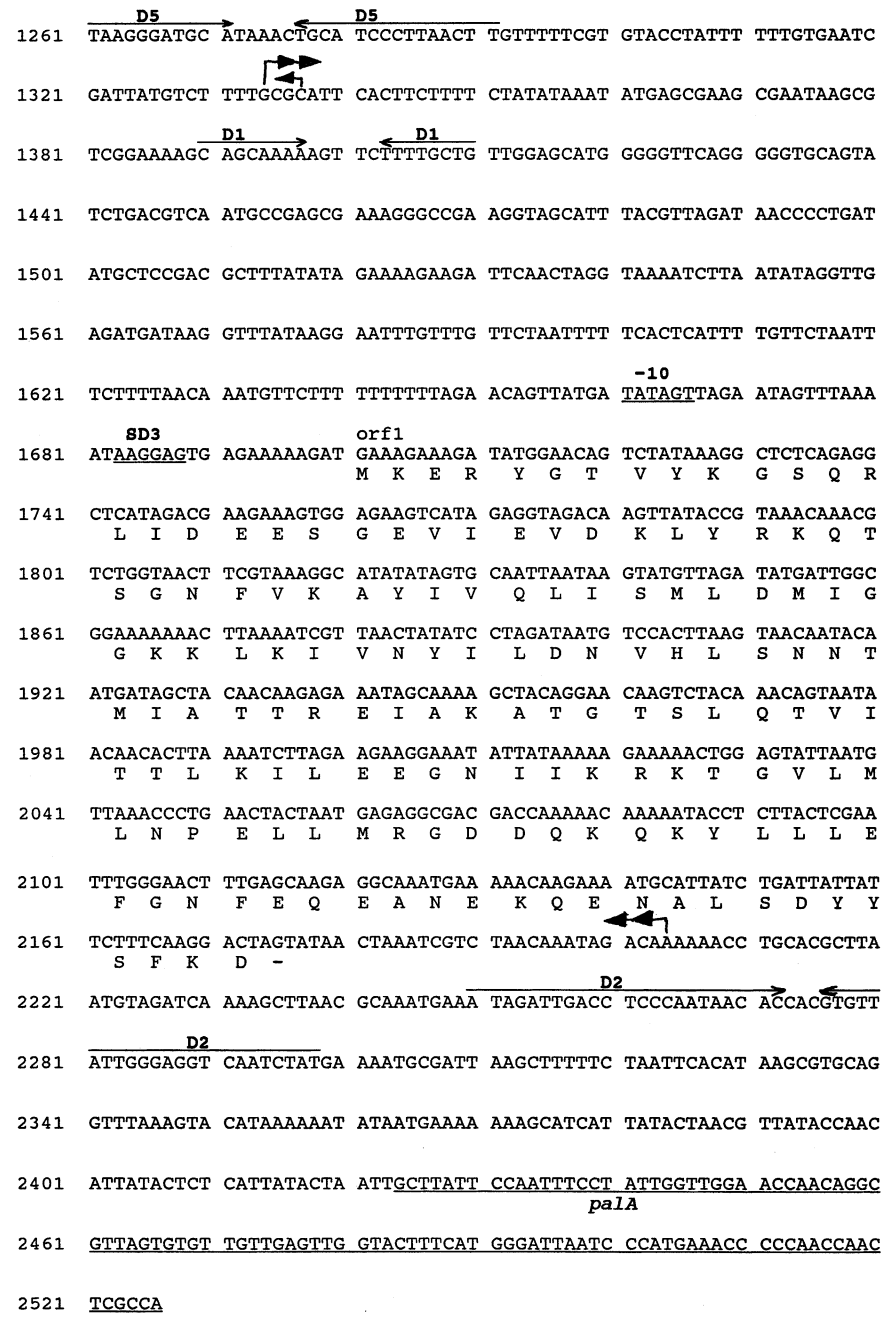

Fig. 3 (*continued*).

homology (97%+) with *erm* sequences found in *S. aureus* Em^R plasmids pE194 [19], pT48 [23] and pE5 [24], the *S. epidermidis* plasmid pNE131 [20], the *Bacillus subtilis* plasmid pIM13 [25], and pPV141 found in *S. chromogenes* [13].

Sequence alignment of the region upstream from

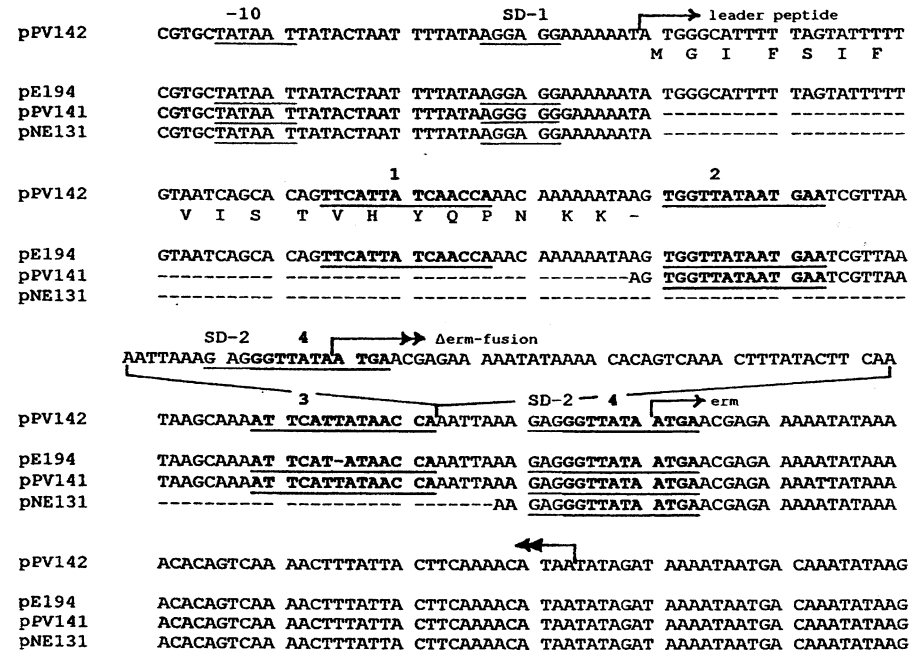

Fig. 4. Sequence alignment of the 5′ flanking region of *erm* genes. Sequences of pE194 (GenBank accession number VO1280; nt 2896C–2659C) and pNE131 (GenBank accession number 12730; nt 728–859) were retrieved from the GenBank Data Base. Sequences of pPV141 (GenBank accession number U82607; nt 303–483) and pPV142 (this study, GenBank accession number AF019140; nt 304–603) were determined in this laboratory. The complementary repeat sequences 1, 2, 3, and 4 [26] are underlined and shown in bold-face letters. The open reading frame encoding the putative fusion polypeptide (*erm*-fusion) is flanked by the double-head arrows. The start codon of the intact *erm* gene product is marked by an arrow. Putative ribosomal binding sites (SD1 and SD2) and −10 consensus promoter sequences are underlined.

the *erm* gene of pPV142 with those of pPV141 and pNE131 showed that, unlike the latter two plasmids, a deletion had not occurred in pPV142 (Fig. 4). Detailed analysis of this 5′ region of ORF2 (*erm*) revealed the presence of the four complementary repeat sequences reported earlier for pE194 [19,26]. Furthermore, comparison of this DNA segment with the corresponding region in pE194 revealed that a 61-bp DNA sequence immediately following the complementary repeat sequence 3 (Fig. 4), and situated between coordinates 446 and 506 (Fig. 3) had been duplicated with 97%+ fidelity between coordinates 507 and 569 in pPV142. The duplication resulted in the occurrence of two copies of the Shine-Dalgarno unit SD2, and part of the 5′ end of the *erm* gene. One consequence of the sequence duplication is the potential synthesis of a 37-residue polypeptide containing the NH_2-terminal end of the *erm* gene product. Similar to pE194 [19], a leader peptide is present in pPV142 and, as a consequence, the translational attenuation mechanism proposed for the inducible expression of *ermC* [27] remains intact for the synthesis of this putative fusion polypeptide. On the other hand, the sequence duplication might relieve the synthesis of the downstream intact *erm* gene from attenuation control. Repeat sequence 3, which could base-pair with the duplicate repeat sequence 4 of the putative fusion polypeptide, is no longer available for base-pairing with the complementary sequence 4 of the intact *erm* gene. Consequently, the expression of *erm* in pPV142 is constitutive. This novel mechanism of circumventing translational attenuation adds another variation to the previously reported genetic rearrangements at the 5′ flanking region of the *erm* gene [13,26,28].

The constitutive expression of *erm* was supported by the results of subculturing experiments with *S. simulans* 13044 showing retention of the constitutive

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